EXHIBIT 6f

antioxidants, glutathione peroxidase, and GSH in humans.[325, 326, 327] Similar activity is expected in all mammals, but milk GSH testing is largely unexplored in rodents due to their size. Bovine whey protein has also been shown to protect mice from 300mg/Kg APAP induced hepatotoxicity as indicated by reduced liver enzymes (glutamate pyruvate transaminase, GPT; alkaline phosphatase, ALP), reduced creatinine, increased BUN, increased antioxidant enzymes (catalase, CAT; superoxidase dismutase, SOD; glutathione peroxidase, GSH-Px) and reduced TBARS.[328]

	Lethality†		
		Paracetamol	
Age (wk)	Control	300 mg/kg	500 mg/kg
2	0	0	0
3	0	0	3
8–10	0	0	7
24–26	0	0	8
32–34	0	0	5
52–54	0	3	9

* In each age group, 10 animals received either dose of paracetamol and four control animals received saline intraperitoneally.
† Number of deaths in 8 h.

Table 8. The Lethality of Paracetamol (APAP) in Mice by Age in CD-1 Mice. The number of mice that die out of 10/group increases with dose of APAP IP and age of mice. In two-week-old mice there was no lethality, no hepatocytic necrosis, and no increase in plasma transaminases.

NTP 1993

A continuous dietary exposure toxicity study was performed by the NTP.[329] These studies included 14-day, 13-week, and 2-year exposures to APAP via fed diets at exposures ranging from 800-12,500PPM, 800-25,000PPM, and 600-6000PPM. For comparability, these dosages are 600PM = 0.06% or ~86mg/Kg to 25,000PM = 2.5% or 3575mg/Kg. The upper dose of 3575mg/Kg in mice converts to a human dosage of 291mg/Kg or a 17.5g dose in a 60Kg reference human. This upper dose resulted in one death in a female mouse during the 13-week, and the 12500PPM (mouse 1788mg/Kg, HED 146mg/Kg) dose also resulted in the death in two male mice during the 13-week study. These studies are also consistent with the expected acute toxicity of humans at dosages of 7.5g or ≥150mg/Kg.

Thomson 1995; Kelava 2010

[325] Ankrah et al. Human breastmilk storage and the glutathione content. J Trop Pediatr. 2000 Apr;46(2):111-3. doi: 10.1093/tropej/46.2.111. PMID: 10822938.
[326] Sari et al. Antioxidant capacity of fresh and stored breast milk: is -80°C optimal temperature for freeze storage? J Matern Fetal Neonatal Med. 2012 Jun;25(6):777-82. doi: 10.3109/14767058.2011.592230. Epub 2011 Aug 1. PMID: 21801121.
[327] Miranda et al. Oxidative status of human milk and its variations during cold storage. Biofactors. 2004;20(3):129-37. doi: 10.1002/biof.5520200302. PMID: 15665383.
[328] Athira et al. Ameliorative potential of whey protein hydrolysate against paracetamol-induced oxidative stress. J Dairy Sci. 2013 Mar;96(3):1431-7. doi: 10.3168/jds.2012-6080. Epub 2013 Jan 9. PMID: 23313001.
[329] National Toxicology Program. NTP Toxicology and Carcinogenesis Studies of Acetaminophen (CAS No. 103-90-2) in F344 Rats and B6C3F1 Mice (Feed Studies). Natl Toxicol Program Tech Rep Ser. 1993 Jan;394:1-274. PMID: 12637965.

Additional studies in mice have shown that propylene glycol is protective against APAP hepatoxicity, due to inhibition of CYP2E1, which also results in reduced loss of GSH and lowered ALT.[330,331] These data support that propylene glycol modifies APAP toxicity by decreasing CYP2E1. These data are also consistent with the observations that lower expression or reduced activity of cytochrome P450 enzymes results in increased resistance to APAP, mimicking the Cyp2e1 -/- and Cyp1a2 -/- mutant mice (Figure 27).

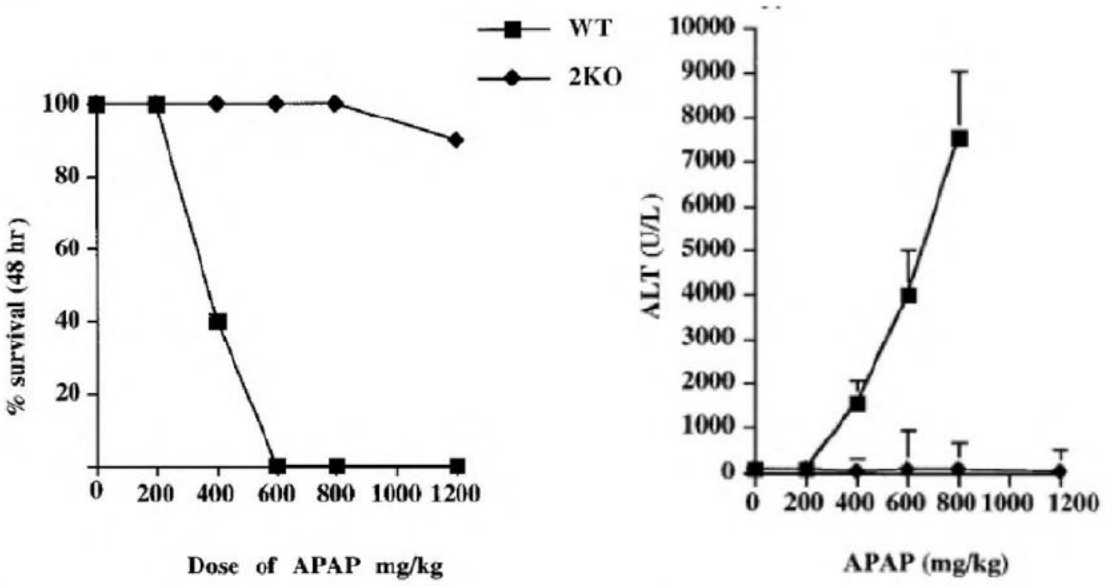

Figure 27. Cytochrome P450 and Acetaminophen (APAP) Toxicity. (Left) Survival of wild-type and double-null mice (Cyp2e1 -/- and Cyp1a2 -/-) after APAP. Mice were treated with increasing doses of APAP (n = 8 per group). Survival was observed 48 h after the challenge dose and percent survival was plotted as a function of challenge dose. (Right) ALT in APAP treated mice. ALT was measured in plasma of wild-type and double-null mice after APAP exposure. Values are means +/- SE from groups of four to six mice. *p <= 0.05 WT vs 2KO mice.[332]

Patel 2001

Patel examined acetaminophen in C57BL/6 mice, giving three dosages IP at 100, 200, or 250mg/Kg results in no significant increase in liver weight or ALT.[333] There were also no significant changes reported in uterine weight, uterine peroxidase activity, or uterine or hepatic progesterone receptor with APAP treatment compared to vehicles. These data indicate that at IP dosages up to 250mg/Kg, for three consecutive days, in C57Bl/6 female mice there is no overt hepatotoxicity. For comparison, the HED "therapeutic" dose of APAP in mice is 205mg/Kg, so these dosages are ~0.5X, ~1X, and ~1.2X HEDs.

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[330] Kelava et al. Influence of small doses of various drug vehicles on acetaminophen-induced liver injury. Can J Physiol Pharmacol. 2010 Oct;88(10):960-7. doi: 10.1139/y10-065. PMID: 20962895.

[331] Thomsen et al. Cytochrome P4502E1 inhibition by propylene glycol prevents acetaminophen (paracetamol) hepatotoxicity in mice without cytochrome P4501A2 inhibition. Pharmacol Toxicol. 1995 Jun;76(6):395-9. doi: 10.1111/j.1600-0773.1995.tb00168.x. PMID: 7479582.

[332] Zaher et al. Protection against acetaminophen toxicity in CYP1A2 and CYP2E1 double-null mice. Toxicol Appl Pharmacol. 1998 Sep;152(1):193-9. doi: 10.1006/taap.1998.8501. PMID: 9772215.

[333] Patel and Rosengren. Acetaminophen elicits anti-estrogenic but not estrogenic responses in the immature mouse. Toxicol Lett. 2001 May 31;122(1):89-96. doi: 10.1016/s0378-4274(01)00352-6. PMID: 11397560.

This study is consistent with an absence of hepatotoxicity with mouse dosages near recommended therapeutic HED.

Leroux 2010

Time and/or dose dependent toxicity was reported by Leroux et al.[334] This study reports that a dose of 10ug given PD1-5 resulted in 38% survival (6/16). Conversion of this dosages is a tenuous estimate, as the dose was not adjusted by pup weight or age, so a PD1 newborn, which is expected to weigh ~1g is given the same dose of a PD5, reported to weigh 2.4 to 3.6 g. This results in newborn pups being treated with 2.4-3.6X the dosage of the PD5, so estimated dosages range from 100mg/Kg for the 100ug dose at PD1, assuming a 1-gram pup, to 2.7mg/Kg for the 10ug dose in a 3.6-gram PD5 pup. The high APAP dose (100ug), given early PD1-5, resulted in increased pup lethality. The low dose of APAP (10ug), given to older pups (PD5-10) was reported to protect the brain, as indicated by reduced sizes of white matter lesions with ibotenate exposure.

Iacob 2019

This study exposed pregnant white mice exposed to acetaminophen (50mg/Kg/day) from GD5-7.[335] After delivery, pups were examined for liver and kidney pathology. Results showed that the number of fetuses born was normal. Pathology of the liver and kidney revealed APAP produced urinary tube necrosis, vascular congestions, and vascular disorders in the kidneys, and vascular congestions, vascular disorders, and hemosiderin deposits in Kupffer cells in the liver. The study concluded that APAP did not reduce fertility at this dose and duration, but it did produce microscopic changes in the liver and kidney pathology in offspring.

Viswanathan 2019

Viswanathan et al[336] gave mice different combinations of a Centella asiatica methanol (CAM) extract and APAP (PO, 500mg/Kg) and measured the levels of oxidative and nitric oxide stress in the brains of mice. The authors also looked at the cytotoxicity and inflammation levels by examining the expression of cytokine mediators in the brain tissue and assessing apoptosis/necrosis. The results showed that APAP decreased ferric reducing antioxidant power and increased reactive nitrogen species in brains both *in vitro* and *in vivo*. CAM extract was able to reduce this effect and increase the scavenging activity. APAP showed the presence of more double positive necrotic/apoptotic cells in astrocytes. The extract was also able to inhibit the expression of pro-inflammatory cytokines and increase the expression of anti-inflammatory cytokines, which helped to decrease damage to the brain cells. Overall, the study suggests that the CAM extract has antioxidant and anti-inflammatory effects against oxidative stress and inflammation in the brain caused by APAP.

4. Studies Examining APAP and Reproductive Toxicity.

A number of studies have investigated reproductive toxicity of fetal exposure to APAP. There is evidence that exposure to toxicants can result in epigenetic alterations, notably DNA methylation. Three areas of

[334] Leroux et al. Neuroprotective effects vary across nonsteroidal antiinflammatory drugs in a mouse model of developing excitotoxic brain injury. Neuroscience. 2010 May 19;167(3):716-23. doi: 10.1016/j.neuroscience.2010.02.042. Epub 2010 Feb 24. PMID: 20188153.

[335] Iacob et al. Consequences of analgesics use in early pregnancy: Results of tests on mice. Sci Total Environ. 2019 Nov 15;691:1059-1064. doi: 10.1016/j.scitotenv.2019.07.212. Epub 2019 Jul 15. PMID: 31466187.

[336] Viswanathan et al. Protection of mouse brain from paracetamol-induced stress by Centella asiatica methanol extract. J Ethnopharmacol. 2019 May 23;236:474-483. doi: 10.1016/j.jep.2019.03.017. Epub 2019 Mar 11. PMID: 30872170.

increasing regulatory activity are reproductive toxicity, developmental toxicity, and neurotoxicity. These are endpoints for which an exposure threshold is assumed. That is, an acceptable level of exposure, below which no toxicity is predicted, can be calculated. Studies have confirmed that specific chemical exposures may be linked to infertility, neurodevelopmental disorders, carcinogenesis, and reproductive disruption of gametes.

Reel 1992

This study examined the reproductive toxicity of APAP in mice.[337] The study authors examined chronic exposure to APAP in chow at dosages of 0, 0.25, 0.5, and 1.0%, which were estimated to result in APAP administration of 0, 357, 715, and 1430 mg/Kg, respectively. A preliminary dose study was also performed and estimated that the oral dosage of APAP that produced lethality had a reported LD10 (14 days) of 1.81% APAP (~2574mg/Kg/day) and an LD50 (14 days) of 4.11% APAP (~5877mg/Kg/day). The 1% dose was reported to reduce litter per pair, live pup weight, weaning (PN28) weight, and mating weights (PN74+/-10). In F1 offspring, this dose also resulted in reduced body weights in males and females, and increased weights in the liver and brain, that were reported as significantly different in females. The F1 males were also reported to have more than a doubling of the percentages, 7.3% versus 16.4%, of abnormal sperm at the 1% dosage. Significant reductions in weight were also reported in weaning females at 0.25%, in males and females at mating at 0.25%, and in weaning and mating males and females at 0.5% APAP in diet. The conclusion of this study reads,

> "APAP was found to be a reproductive and systemic toxicant in Swiss CD-I mice, albeit at relatively high exposure levels."

While this statement is largely driven by the mg/Kg dosing, it should again be remembered that the 0.25%, 0.5%, and 1% APAP diets can be converted from mouse mg/Kg dose to a HED, and results in dosages of 30.5, 58.1, and 116.3mg/Kg, respectively. The upper dose for a reference 60Kg human, results in a dose of 6975mg, or approximately 7 grams. As expected, this "relatively high exposure" is remarkably near the 7.5g acute toxic dose in humans using the mouse-human conversion (Table 1).

Holm 2015; Holm 2016

In utero exposure to APAP can also impact reproductive cells and tissues in the offspring. A study by Holm et al. (2015) first reported aniline conversion into APAP and also indicated that APAP impacted anal-genital-distance (AGD), consistent with fetal androgen deficiency during external genitalia differentiation.[31] The study also performed testing on NCI-H295R human adrenocortical carcinoma cell line to examine human androgen production *in vitro* and reported APAP was anti-androgenic. Specifically, steroids were analyzed after exposure for 48h to the following concentrations of 0, 0.1, 0.314, 1, 3.14, 10, 31.4, 100, 314, and 1000μM APAP. APAP increased pregnenolone (EC50=146μM; P=.001) and decreased hormone levels downstream from progesterone: 17a-hydroxyprogesterone (EC50=43 μM; P=.04), androstenedione (EC50=289 mM; P=.001) and testosterone (EC50=97μM; P=.007). Holm et al. (2016) also examined the impact of APAP on female offspring with *in utero* exposure to APAP.[338] This study also observed changes in AGD in female offspring, with the majority occurring at the high dose

[337] Reel et al. Reproductive toxicity evaluation of acetaminophen in Swiss CD-1 mice using a continuous breeding protocol. Fundam Appl Toxicol. 1992 Feb;18(2):233-9. doi: 10.1016/0272-0590(92)90051-i. PMID: 1601223.

[338] Holm et al. Intrauterine Exposure to Paracetamol and Aniline Impairs Female Reproductive Development by Reducing Follicle Reserves and Fertility. Toxicol Sci. 2016 Mar;150(1):178-89. doi: 10.1093/toxsci/kfv332. Epub 2016 Jan 5. PMID: 26732887.

(150mg/Kg), but both dosages (50 and 150mg/Kg) resulted in reduced primordial and growing ovarian follicles. The 50mg/Kg is referenced by the authors as the dose allowed to be taken by pregnant women, and this statement is consistent with the HED in mice. APAP exposure was associated with reduced fertility (% full term pregnancies) and reduced litter size (pups / dam) at 6 months but not 10 months of age. APAP also reduced gonocyte numbers, decreased mitotic activity, with a proposed mechanism of blocking DNA replication, and reduced stem cell proliferation at both the low and high dosage.

The authors conclude that APAP poses a significant risk to the reproductive health of all offspring regardless of sex.

Rossitto Jan. 2019; Rossitto Aug. 2019

A pair of studies on male[339] and female[340] offspring with *in utero* exposure to APAP (30mg/Kg) were performed by Rossitto et al. using CD-1 mice. The latter study used a combination of APAP with ibuprofen, but the former study examined APAP individually as well. The authors reported that exposure to APAP during the period gonadal development can lead to problems in male reproductive cell growth and development. This can result in delayed maturation of cells in the testes and a decrease in the number of sperm produced. They observed reduced sperm count and motility in adult males exposed to APAP *in utero*. These findings suggest that using APAP during this critical time may harm germ-line development, and these adverse effects could be passed on to future generations.

5. Other APAP Mouse Studies

Lee 2015

In a study by Lee et al. there was no reported toxicity on airway hyper-responsiveness (AHR) following APAP exposure during pregnancy or lactation.[341] It is important to note that this study by Lee et al. only dosed at 5mg/Kg, which is well below the therapeutic or toxic dose in mice. This dose selection likely stems from not converting the human mg/Kg dose to a HED in mice (see Table 1). For this example, a recommended 10mg/Kg human pediatric dosage produces a HED of 123mg/Kg for mice, so the tested 5mg/Kg would produce a HED of ~60mg/Kg in mice.

D. Rat Studies on APAP: Causality and Neurodevelopmental Toxicity

The following rat studies were reviewed in regard to general, reproductive, developmental, or neurodevelopmental toxicity of APAP in rats (Table 9). The strategy for identifying relevant studies is described in Appendix B.

Author	Year	Strain	Dose (mg/Kg)
Lubawy and Garrett	1977	Sprague-Dawley	0, 125, 250
Mancini et al.	1980	Sprague-Dawley	1200 - 2350 (LD50)

[339] Rossitto et al. Intergenerational effects on mouse sperm quality after in utero exposure to acetaminophen and ibuprofen. FASEB J. 2019 Jan;33(1):339-357. doi: 10.1096/fj.201800488RRR. Epub 2018 Jul 6. PMID: 29979629.

[340] Rossitto et al. In utero exposure to acetaminophen and ibuprofen leads to intergenerational accelerated reproductive aging in female mice. Commun Biol. 2019 Aug 13;2:310. doi: 10.1038/s42003-019-0552-x. PMID: 31428698; PMCID: PMC6692356.

[341] Lee et al. Perinatal paracetamol exposure in mice does not affect the development of allergic airways disease in early life. Thorax. 2015 Jun;70(6):528-36. doi: 10.1136/thoraxjnl-2014-205280. Epub 2015 Apr 3. PMID: 25841236; PMCID: PMC4453715.

Lin and Levy	1983	Lewis	0, 15, 300
Momma & Takeuchi	1983	Wistar	0, 10, 100, 1000
Harris et al	1988	Sprague-Dawley	n/a
Harris et al	1989	Sprague-Dawley	0, 250, 500μM
Stark et al.	1989	Sprague-Dawley	0, 250, 500μM
Stark et al.	1990	Sprague-Dawley	0,100,250,500,1000μM
Weeks et al.	1990	Sprague-Dawley	300μM
Gandy et al.	1990	Sprague-Dawley	0, 500, 1000, 1500
NTP	1993	F344/N	0, 80, 160, 310, 620, 1250
Ying & Lou	1993	Unspecified	0, 250, 500, 1000
Micheli et al.	1994	Wistar	0, 3000
Lister & McLean	1995	Unspecified	0, 125, 500
Lister & McLean	1997	Wistar	0, 60, 125, 500, 1000
Ratnasooriya & Jayakody	2000	Crossbred Albino	0, 500, 1000
Beck et al.	2001	Sprague-Dawley	0, 125, 250, 500
Ahmed et al.	2003	Sprague-Dawley	0, 500
Neto et al.	2004	Wistar	0, 125, 500, 1500
Oyagbemi et al.	2009	Wistar	0 or 3000
Posadas et al.	2010	Sprague-Dawley	0, 250, 500
Kristensen et al.	2011	Wistar	150, 250 and 350
Dean et al.	2012	Sprague Dawley	0, 40
Blecharz-Klin et al.	2013	Wistar	0, 10, 50
Oyedeji et al.	2013	Albino	0 & 7.5
Axelstad et al.	2014	Wistar	0, 350
Blazquez et al.	2014	Wistar	0, 400mg/Kg by prepregnant weight
Blecharz-Klin et al. (a & b)	2015	Wistar	0, 5, 15
Lichtensteiger et al.	2015	Wistar	0, 360
Blinova et al.	2016	Unspecified Albino	0, 500
Dean et al.	2016	Wistar	0, 350
Johansson et al.	2016	Wistar	0, 360
Blecharz-Klin et al.	2017	Wistar	0, 5, 15
Onaolapo et al.	2017	Wistar	0, 800
Axelstad et al.	2018	Wistar	0, 360
Blecharz-Klin et al.	2018	Wistar	0, 5, 15
Chen et al.	2018	Wistar	0, 50, 300
Hurtado-Gonzalez et al.	2018	Wistar	0, 350
Saeedan et al.	2018	Wistar	0, 50

Toyoda et al.	2018	F344 lean Zucker obese Zucker	0, 4.4, 13.8, 44.2, 142.1, 490.8 0, 4.7, 14.6, 46.5, 152.8, 521.9 0, 5.5, 17.3, 55.4, 169.2, 539.9
Blecharz-Klin et al.	2019	Wistar	0, 5, 15
Motawi et al.	2019	Wistar	0, 400
Vigo et al.	2019	Wistar	0, 100
Aleixo et al.	2020	Wistar	0, 350
Díaz-Estrada et al.	2020	Wistar	0, 100
Klein et al.	2020	Wistar	0, 350
Koehn et al.	2020	Sprague-Dawley	0, 7.5, 15, 30
Koyuncuoglu et al.	2020	Wistar	0, 3000
Lalert et al.	2020	Wistar	0, 200
Oksuz et al.	2020	Wistar	0, 20, 500
Pereira et al.	2020	Wistar	0, 350
Yilmaz et al.	2020	Wistar	0, 50, 125, 250, 500
Koehn et al.	2021	Sprague-Dawley	0, 7.5, 15, 30
Rigobello et al.	2021	Wistar	0, 35, 350
Suda et al.	2021	Sprague Dawley	0, 70.56
Taqa et al.	2021	Unspecified Albino	0, 50
Blecharz-Klin et al.	2022	Wistar	0, 5, 15
Chen et al.	2022	Sprague–Dawley	0, 100, 300
Herrington et al.	2022	Sprague Dawley	0, 51.97
Lalert et al.	2023	Sprague Dawley	0, 200

Table 9. Rat Studies Reviewed for General and Neurodevelopmental Toxicity. The human-rat conversion provided in Table 1 indicates a 1g dose in a 60Kg adult (16.6mg/Kg) when converted to a rat dose produces a HED of ~103mg/Kg. Based on ED_{50} data and HED calculations, a rat dose of ~100-150 mg/Kg is therapeutic, see Human Equivalent Dosages. Several studies report maternal and developmental impacts below this "therapeutic" HED. Oral dosages of 1000mg/Kg are reported to be tolerated, as described below. IP dosages ≥ 800mg have been reported to produce toxicity in rats, which varies by rat strain and animal age.

1. Studies examining whether exposure to acetaminophen caused behavioral changes.

Several studies investigated whether exposure to acetaminophen caused behavioral changes and/or associated biological changes in the brain.

Dean 2012

This study examined the effect of early postnatal APAP exposure on neuropathology and behavior.[342] As discussed above, APAP is a COX inhibitor that inhibits prostaglandins. During development, the cerebellum has a high expression of prostaglandin receptors. This study showed that inhibition of

[342] Dean et al. Prostaglandin E2 is an endogenous modulator of cerebellar development and complex behavior during a sensitive postnatal period. Eur J Neurosci. 2012 Apr;35(8):1218-29. doi: 10.1111/j.1460-9568.2012.08032.x. PMID: 22512254; PMCID: PMC3534986.

prostaglandins in rat pups during early postnatal life altered cerebellar Purkinje cell development, initially resulting in increased dendritic growth in both sexes. APAP was shown to increase spinophilin, which was used as a marker for dendritic spines in the study. APAP specifically increased spinophilin in the cerebellar cortex of pups treated from PN7 to PN13 at a dosage of 200ug (~40mg/Kg). This impact on dendritic spines resulted in later cerebellar atrophy in males only. In addition, social interaction and sensory threshold were altered in males. The dose of 40mg/Kg would produce an adult human equivalent dose (HED) of 6.5 mg/KG, which would be about 400 mg in a 60Kg adult. The authors report that this dosage,

> is the rectal dose of acetaminophen used in settings such as pediatric emergency rooms and intensive care units.

This study proposes that prostaglandins, produced by COX enzymes, are important for brain development, and especially in the cerebellum, which is involved in many different brain functions. The authors propose that there is an increasing awareness of cerebellar pathology in disorders such as ASD, and they suggest that the results from the study support the impact of the cerebellum, and thereby APAP, on neurodevelopmental disorders such as ASD.

Blecharz-Klin 2013, Blecharz-Klin 2017

In the 2013 study, three-month old male Wistar rats were given either 0, 10, or 50mg/kg/day of acetaminophen for eight weeks.[343] The modified Morris water maze task was used to measure impacts on behavioral performance and learning. There were no significant differences between the groups in the water maze test. During the second day of training, working memory improved in the 10 and 50mg/Kg dosages. The study also showed an increase in the mean swimming speed of treated animals. High Performance Liquid Chromatography (HPLC) was used to determine the brain concentrations of neurotransmitters and metabolites. There were significant differences in monoamines and metabolites between the experimental groups. The authors conclude that the experiment shows that eight weeks of treatment with APAP results in changes in behavior and working memory in rats. The acetaminophen dosages of 10mg/Kg and 50mg/Kg used in this study have HEDs of 1.6mg/Kg and 8mg/Kg, respectively. This would be about 100mg and 500mg for a 60Kg adult, which is 10-50% of a single extra-strength dose (1000mg).

In the 2017 study, the investigators examined the behavioral impacts on Wistar rats that resulted from exposure during pregnancy and lactation to APAP (0, 5, 15mg/kg).[344] Specifically, they examined spatial learning and memory consolidation and the level of physical and exploratory activity. They also performed analysis (by HPLC) on brain concentrations of monoamines, metabolites, and amino acids. They reported no difference in motor performance with APAP exposure, but the lower dose of APAP (5mg/Kg) improved spatial memory and altered exploratory behavior. They also reported a decrease in motor activity and head-dipping at this exposure, and an increase in the number of crossings in the Water Maze at this dose. There were also reported changes in the amino acid levels in the hippocampus and cortex caused by APAP exposure. The authors conclude that exposure to APAP during fetal or early postnatal life dose-

[343] Blecharz-Klin et al. Paracetamol--the outcome on neurotransmission and spatial learning in rats. Behav Brain Res. 2013 Sep 15;253:157-64. doi: 10.1016/j.bbr.2013.07.008. Epub 2013 Jul 11. PMID: 23850354.

[344] Blecharz-Klin et al. Paracetamol - Effect of early exposure on neurotransmission, spatial memory and motor performance in rats. Behav Brain Res. 2017 Apr 14;323:162-171. doi: 10.1016/j.bbr.2017.01.051. Epub 2017 Feb 3. PMID: 28163096.

dependently alters cognitive functions, spatial working memory, motor performance, and there were significant alterations of neurotransmission in rats. These low dose studies have resulted in a proposed model in which APAP improves memory and/or learning at low concentrations. Specifically, the dosages used in this study were a fraction of a daily therapeutic HED, with the tested range (5-15mg/Kg) producing HEDs of 0.8-2.4mg/Kg; this results in reference human (60Kg) dosages of 50-150mg, or only 5-15% of a single recommended 1000mg dosage.

Onaolapo 2017

This study examined whether APAP exposure produced injury in kidney, liver, and cortical neurons.[345] The experimental exposure was 800 mg/kg/day (IP) for three consecutive days. As indicated previously, this dosage and route of exposure is considered an APAP overdose, based on both HED (7.7g, which is greater than 7.5g, the dose identified as causing acetaminophen poisoning in humans) and experimental findings in this and other rat studies. At this dosage, the authors report significantly reduced body weight, reduced locomotion, increased grooming, decreased spatial memory, and increased anxiety, with significant changes in clinical chemistry, including elevated AST, ALT, urea, and creatinine.

Blecharz-Klin 2018

This study investigated the effect of perinatal exposure to APAP on social behavior and levels of brain-derived neurotrophic factor (BDNF) in rats.[346] Wistar rats were exposed *in utero* during pregnancy and early life to low doses of APAP (0, 5, 15mg/kg). Animals exposed to APAP exhibited a lower total frequency of social interactions and greater pinning behavior, and rats from the 15mg/kg group exhibited a greater interest in objects in the novel object recognition tests. The striatum of rats exposed to APAP had a two-fold decrease in levels of BDNF in the striatum portion of the brain. The striatum is known to be involved in the integration of social information, cognitive processes and reward coding in the brain. And BDNF promotes the maturation, survival, and function of neurons.

Saeedan 2018

To examine the interactions between fever and APAP treatment and ASD risk, these investigators developed an experimental rat model of vaccine-induced fever with subsequent treatment for fever reduction by APAP.[347] This study is significant because it addresses the relative risk of fever and APAP to produce ASD, or neurodevelopmental toxicity. This is commonly referred to as confounding by indication in epidemiology studies, where a medical treatment may be associated with causing damage, but the damage can actually be caused by the indication or underlying illness being treated.

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[345] Onaolapo et al. l-Methionine and silymarin: A comparison of prophylactic protective capabilities in acetaminophen-induced injuries of the liver, kidney and cerebral cortex. Biomed Pharmacother. 2017 Jan;85:323-333. doi: 10.1016/j.biopha.2016.11.033. Epub 2016 Nov 23. PMID: 27889232.

[346] Blecharz-Klin et al, Early paracetamol exposure decreases brain-derived neurotrophic factor (BDNF) in striatum and affects social behaviour and exploration in rats, Pharmacology, Biochemistry and Behavior 168 (2018) 25–32, DOI:/10.1016 /j.pbb.2018.03.044

[347] Saeedan et al. Effect of early natal supplementation of paracetamol on attenuation of exotoxin/endotoxin induced pyrexia and precipitation of autistic like features in albino rats. Inflammopharmacology. 2018 Aug;26(4):951-961. doi: 10.1007/s10787-017-0440-2. Epub 2018 Jan 11. PMID: 29327281.

In this study, pups were treated with measles mumps rubella (MMR) vaccine, diphtheria tetanus and pertussis (DPT) vaccines, or lipopolysaccharide (LPS), individually and with subsequent APAP treatment at 50 mg/kg. Control and treated pups were evaluated for growth and development. The authors report a correlation between social-behavioral testing results and inflammatory markers in the pups. They report that all treatment groups showed significant alteration in the behavioral changes and increased oxidative markers (TBARS) and inflammatory markers. Exposure to APAP (50 mg/kg sc.) on PND5 resulted in the highest increase in TBARS in examined brain tissue. APAP was also reported to produce significant and time dependent impacts on eye opening, locomotor activity, olfactory discrimination, and on entries in the elevated plus maze. Based on the results that all exposures resulted in increased pathology and increased oxidative stress, the authors propose that both the indication (fever) and treatment (APAP) are causative. Specifically, they propose that dysregulated immune/inflammatory circuits and/or increased catecholamine in the brain (due to sulfur and/or amine disruptions induced by APAP) can increase neurobehavioral and neurotoxic effects in newborn brains with increased underlying susceptibility.

Klein 2020

This study examined whether APAP caused behavioral changes in Wistar rats exposed *in utero* to APAP (350mg/Kg).[348] Treatments were via gavage (350 mg/kg/day) or water from GD6 until delivery. The general toxicity endpoints included body weight and food intake. Behavioral evaluation occurred on PND10 (nest seeking test), PND27 (behavioral stereotypy), PND28 (three chamber sociability test and open field) and PND29 (hot plate and elevated plus-maze). Reduced glutathione (GSH) and brain derived neurotrophic factor (BDNF) levels were also quantified in the prefrontal cortex and hippocampus on PND22. Exposure to APAP impaired nest seeking behavior and decreased rostral grooming in the elevated plus maze. Alterations in glutathione levels or BDNF were not observed in the prefrontal cortex or hippocampus. Behaviors on the hot plate and sociability tests were unaffected by APAP. The authors conclude that the data support APAP as a neurotoxicant.

Rigobello 2021

Rigobello et al.[349] proposed to examine if perinatal exposure to APAP, at human-relevant doses, could alter the brain and behavior of offspring. Wistar dams were gavaged daily with water or APAP (35 mg/kg or 350 mg/kg) from GD6-PND21. Behavior testing occurred at PND10 (nest seeking test), PND27 (behavioral stereotypy), and PND28 (three-chamber sociability test and open field). Compared to control animals, perinatal exposed males had augmented stereotyped behavior and ambulation in an open-field test. They also reported a reduction in exploratory behavior in the three-chamber sociability test (high dose). APAP decreased the levels of glutathione (low dose, hippocampus). The authors propose that use of APAP during pregnancy and breastfeeding is a potential risk factor for neurological disorders.

Suda 2021

[348] Klein et al. Gestational exposure to paracetamol in rats induces neurofunctional alterations in the progeny. Neurotoxicol Teratol. 2020 Jan-Feb;77:106838. doi: 10.1016/j.ntt.2019.106838. Epub 2019 Oct 20. PMID: 31644948.

[349] Rigobello et al. Perinatal exposure to paracetamol: Dose and sex-dependent effects in behaviour and brain's oxidative stress markers in progeny. Behav Brain Res. 2021 Jun 25;408:113294. doi: 10.1016/j.bbr.2021.113294. Epub 2021 Apr 6. PMID: 33836167.

These study authors exposed pregnant rats to APAP (14.7mg/Kg) from PND4-10.[350] This dose was described as a pediatric human dosage (mg/Kg) but was not converted to a HED. The antioxidants cysteine and mannitol were included to prevent accumulation of NAPQI. Western diet, ampicillin (15 mg/Kg), and killed bacteria (1E6) with antibiotics were also examined as potential stressors. The antioxidants were reported to not correct the impact of APAP on behavior. Treatment with acetaminophen was associated with a significant increase in rearing, an asocial behavior, regardless of whether the animals were exposed to other oxidative stress factors or antioxidants. The authors suggest,

> …the inclusion of multiple oxidative stress inducers in the model did not significantly alter the outcome, suggesting that the neurodevelopmental effects we observed are, at least to some extent, independent of oxidative stress.

Measurements of oxidative stress, glutathione, or products produced by NAPQI were not determined in this study. The study authors report: "it is apparent that the currently approved use of acetaminophen in infants and children would never have passed pre-clinical testing if the drug were evaluated based on current standards." And the authors conclude:

> Given current evidence and the fact that the primary target organ for the drug is the brain, it is now quite apparent that proof of absence of damage to the liver should never have been considered adequate proof of safety.

Díaz-Estrada 2021

Based on the endocrine and neuroendocrine impacts of APAP on the developing rat brain, a study was performed to examine the impact on sexual interactions following *in utero* APAP treatments.[351] This study addressed what was termed the sexual partner preference (SPP) of adult male rats treated with APAP. The authors hypothesized that inhibition of cyclooxygenase by APAP during brain development would result in increased homosexual SPP. Treatment with APAP was from GD16-20 of pregnancy, with pregnant female rats receiving either saline or APAP (50mg/Kg, SC) every 12 h. On PND60, half of the male offspring were exposed to sexual experience with receptive females during 5 trials, while the other half remained sexually naive. On PND90, all male offspring were tested for SPP with one sexually receptive female and one stud male. The authors report that APAP-experienced males displayed SPP for females, but APAP-naive males failed to display heterosexual SPP (Figure 28). The authors conclude that APAP disrupted neurotypical heterosexual behavior in rats (nature mechanisms), but early heterosexual experiences restored heterosexual SPP (nurture mechanisms). The dose tested in this study produces a HED of ~8mg/Kg, or about 500mg in a 60Kg adult, or half the recommended therapeutic dose of 1000mg.

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[350] Suda et al. Therapeutic doses of acetaminophen with co-administration of cysteine and mannitol during early development result in long term behavioral changes in laboratory rats. PLoS One. 2021 Jun 25;16(6):e0253543. doi: 10.1371/journal.pone.0253543. PMID: 34170958; PMCID: PMC8232535.

[351] Díaz-Estrada et al. Nature and nurture of sexual partner preference: Teachings from prenatal administration of acetaminophen in male rats. Horm Behav. 2020 Aug;124:104775. doi: 10.1016/j.yhbeh.2020.104775. Epub 2020 Jun 6. PMID: 32422195.

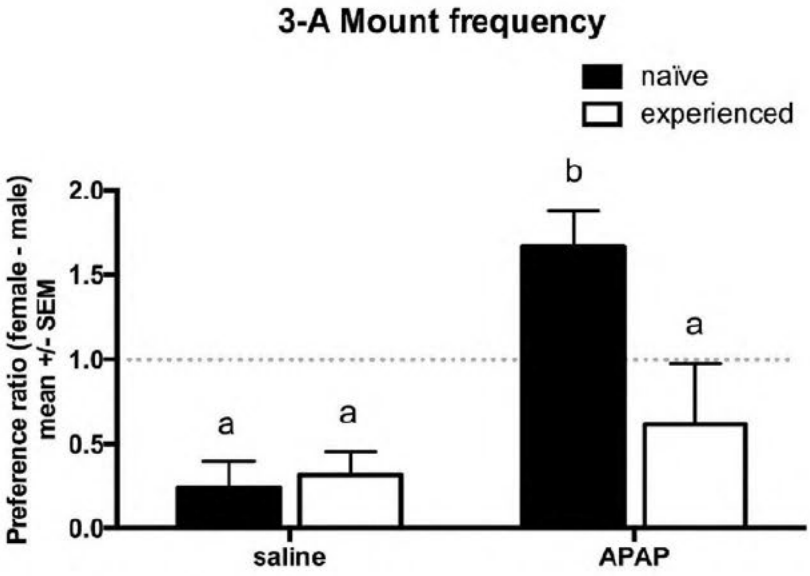

Figure 28. Preference Ratio of Copulatory Sexual Behaviors. Ratios of 1.0 indicate no preference, whereas deviation to 2 indicatesmale preference, and deviation to 0 indicate female preference. Different letters indicate significant findings ($p < .05$) between groups. Naïve in utero exposed APAP males show increased homosexual mounting.

2. Studies examining whether exposure to acetaminophen caused biological changes in the brain.

Several studies investigated whether exposure to acetaminophen caused biological changes in the brain. (As noted above, some of the studies in section 1 also examined biological changes in the brain.)

Harris 1988, Harris 1989

Harris in 1988 used the rat embryo culture model to show that glutathione (GSH) was a modifier of teratogenesis for diverse chemicals, including valproic acid (VPA).[352] The interaction between VPA and glutathione (GSH) is of particular interest because VPA exposure has been recognized as causing behaviors representing ASD, and VPA-exposed rodents are regularly used to model ASD. These researchers then examined the impact of APAP on neurulation staged rat embryos in culture.[353] Briefly, the addition of APAP (250 or 500μM) resulted in abnormal closure of the anterior neuropore of cultured rat embryos. Failure to close the anterior neuropore results in either exencephaly or anencephaly, both of which are neural tube defects (NTDs).

Micheli 1993

[352] Harris et al. Glutathione status and the incidence of neural tube defects elicited by direct acting teratogens in vitro. Teratology. 1988 Jun;37(6):577-90. doi: 10.1002/tera.1420370607. PMID: 3135633.
[353] Harris et al. Abnormal neurulation induced by 7-hydroxy-2-acetylaminofluorene and acetaminophen: evidence for catechol metabolites as proximate dysmorphogens. Toxicol Appl Pharmacol. 1989 Dec;101(3):43
2-46. doi: 10.1016/0041-008x(89)90192-0. PMID: 2603160.

This study examined the effect of acetaminophen on glutathione levels in the brain.[354] Male albino Wistar rats were treated with APAP. One group (n=9) was given 3 gm/kg of APAP while the control group (n=8) received only the vehicle (2% gum arabic in saline). The rats were decapitated 6 hours after administration to avoid the influence of circadian rhythm on GSH levels. This time limit was chosen because peak concentrations of APAP in rat brains occur approximately 6 hours after dosing. APAP and GSH concentrations were determined in the following areas: hypothalamus, corpus striatum, hippocampus, limbic area, pons, medulla oblongata, cerebral cortex, and cerebellum. The authors reported that large reductions in GSH levels were seen in the hypothalamus (- 20%), medulla oblongata (- 14%), and cerebral cortex (- 24%). They also propose that a 20% to 30% decrease of GSH levels is considered significant and able to result in cell injury or cell death if GSH is not restored.

Posadas 2010

This study examined whether exposure to APAP caused neurotoxic effects in rats.[355] The study authors report:

> The data presented here establish, for the first time, a direct neurotoxic action by APAP both in vivo and in vitro in rats at doses below those required to produce hepatotoxicity and suggest that this neurotoxicity might be involved in the general toxic syndrome observed during patient APAP overdose...

Experimentally, the study used both *in vitro* culture conditions and *in vivo* treatment of animals. The impact of 250 and 500mg/Kg APAP in rats was reported to result in concentrations from 1-2mM in plasma and 1-3mM in cerebral spinal fluid. These concentrations were also reported to be neurotoxic both *in vitro* and *in vivo* (Figure 29). The results of acute treatment with APAP were shown to produce apoptosis and DNA damage in the brains of exposed animals.

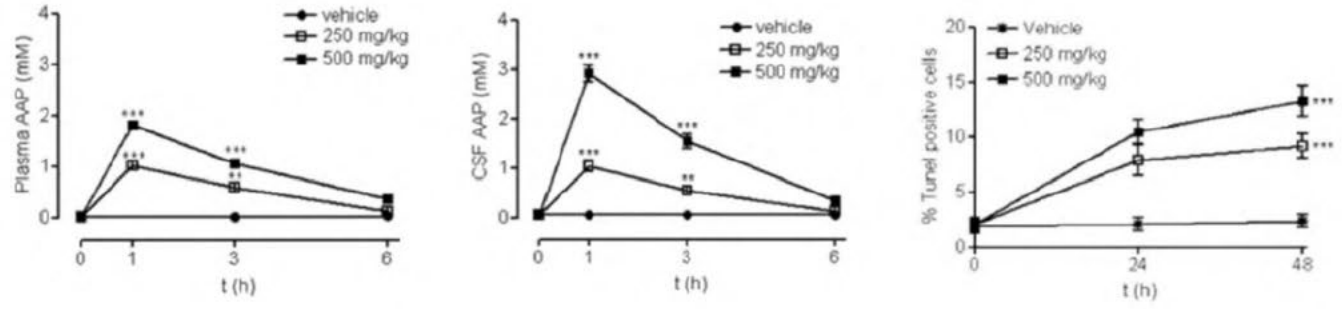

Figure 29. Acetaminophen (AAP) Dosing and Plasma and Cerebral Spinal Fluid Concentrations. Dosages of 250 or 500mg/Kg were administered to rats. The resulting concentrations of AAP/APAP are in the mM range for both (Left) plasma and (Middle) cerebral spinal fluid (CSF). (Right) Tunel positive cells are undergoing apoptosis, with increased DNA fragmentation in animals dosed at 250 and 500mg/Kg.[356]

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[354] Micheli et al. Effect of acetaminophen on glutathione levels in several regions of the rat brain. Curr Ther Res 1993;53:730–6. https://doi.org/10.1016/S0011-393X(05)80745-3

[355] Posadas et al. Acetaminophen induces apoptosis in rat cortical neurons. PLoS One. 2010 Dec 10;5(12):e15360. doi: 10.1371/journal.pone.0015360. PMID: 21170329; PMCID: PMC3000821.

[356] Posadas et al. Acetaminophen induces apoptosis in rat cortical neurons. PLoS One. 2010 Dec 10;5(12):e15360. doi: 10.1371/journal.pone.0015360. PMID: 21170329; PMCID: PMC3000821.

Blecharz-Klin 2014

This study examined whether exposure to APAP caused effects on concentrations of amino acids in the prefrontal cortex, hippocampus, hypothalamus, and striatum of Wistar rats, as measured using High Performance Liquid Chromatography.[357] The study authors report a decrease in the concentration of amino acids in the striatum (glutamine, glutamic acid, taurine, alanine, aspartic acid) and hypothalamus (glycine) between APAP treated rats compared to controls. In the prefrontal cortex, APAP also resulted in increased levels of γ-aminobutyric acid (GABA). The authors conclude that long-term treatment with APAP has a significant effect on the levels of amino acids in the striatum, hypothalamus, and prefrontal cortex of rats.

Blecharz-Klin 2015a, 2015b

These study authors examined whether early life administration of APAP would affect the levels of neurotransmitters in the spinal cord of rat pups.[358] The Wistar rats were exposed to APAP at 0, 5, or 15mg/Kg per day prenatally and for two months postnatal. High Performance Liquid Chromatography was used to determine the level of monoamines, metabolites, and amino acids in the spine of rats. The study authors reported that during this critical period of neurodevelopment, the content of aspartic and glutamic acids in the spine were increased by APAP. A similar study by this group also indicated that APAP also alters the dopaminergic system in the medulla oblongata.[359] The acetaminophen dosages of 5mg/Kg and 15mg/Kg used in this study have HEDs of 0.8mg/Kg and 2.4mg/Kg, respectively. This would be about 50mg and 150mg for a 60Kg adult, which is between 5% and 15% of single extra-strength dose (1000mg).

Lichtensteiger 2015

This research group examined the impact of APAP on Wistar rats during sexual brain differentiation. Specifically, gene expression was analyzed by real-time RT-PCR in the preoptic area and ventromedial hypothalamus animals exposed to APAP or various mixtures of endocrine disrupting chemicals (EDCs).[360] This included an androgen mix (A-mix) and estrogen mix (E-mix) and a combination mixture (A+E+APAP = AEP-mix). Sex and region-specific gene expression patterns in brains from offspring were reported. The A or E-mix was administered by oral gavage to rat dams from gestation day 7 until weaning. The AEP-mix or APAP alone was administered from GD13–GD19. The authors conclude that APAP produces sex and region-specific gene expression patterns in developing rat brain during a critical, sex hormone-sensitive period. The dosage used in these series of studies (350mg/Kg) produces a HED of ~56.5mg/Kg (3.4g in a 60 Kg adult), which is 3.4X a single 1000mg human therapeutic dose, and somewhat less than the recommended limit of 4000 mg in 24 hours.

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[357] Blecharz-Klin et al. Paracetamol impairs the profile of amino acids in the rat brain. Environ Toxicol Pharmacol. 2014 Jan;37(1):95-102. doi: 10.1016/j.etap.2013.11.004. Epub 2013 Nov 13. PMID: 24316461.

[358] Blecharz-Klin et al. Effect of prenatal and early life paracetamol exposure on the level of neurotransmitters in rats--Focus on the spinal cord. Int J Dev Neurosci. 2015 Dec;47(Pt B):133-9. doi: 10.1016/j.ijdevneu.2015.09.002. Epub 2015 Sep 25. PMID: 26390956.

[359] Blecharz-Klin et al. Developmental exposure to paracetamol causes biochemical alterations in medulla oblongata. Environ Toxicol Pharmacol. 2015 Sep;40(2):369-74. doi: 10.1016/j.etap.2015.07.001. Epub 2015 Jul 4. PMID: 26233562.

[360] Lichtensteiger et al. Differential gene expression patterns in developing sexually dimorphic rat brain regions exposed to antiandrogenic, estrogenic, or complex endocrine disruptor mixtures: glutamatergic synapses as target. Endocrinology. 2015 Apr;156(4):1477-93. doi: 10.1210/en.2014-1504. Epub 2015 Jan 21. PMID: 25607892.

Blecharz-Klin 2019

This study examined the interaction of perinatal exposure to APAP with the hypothalamus.[361] The interest in hypothalamic changes were reported to stem from the influence of APAP on fever, which is influenced by this region of the brain. Wistar rats were exposed during pregnancy and lactation to low doses of APAP (0, 5, 15mg/kg). The investigators measured the resulting levels of monoamines, metabolites, and amino acids in the hypothalamus of offspring. The concentration of noradrenaline in the hypothalamus was found to be different between groups. Dopamine and homovanillic acid were increased in the hypothalamus of animals treated at 5mg/Kg. There was also a significant difference in the concentration of glutamic acid at this dose. The authors conclude that perinatal exposure to APAP alters neurotransmitters and metabolites in the brain; specifically, dopaminergic, noradrenergic, and glutamic acid in the hypothalamus. They also propose that this study defines one of the mechanisms by which APAP can interfere with normal reproductive-testicular function, i.e., disturbance of the catecholamine-dependent hypothalamus-testicular pathway.

Motawi 2019

This study examined biological changes in the brain caused by exposure to APAP at therapeutically relevant dosages.[362] In this study, male Wistar albino rats were treated with APAP (400 mg/kg, via gavage) for 28 days. Results indicated significant alteration in brain neurotransmitters and thyroid-hormones, including decreased thyroid releasing hormone (TRH), thyroid stimulating hormone (TSH), triiodothyronine (T3), and thyroxine (T4). There were also significant changes in oxidative stress markers in the brain, including significant decreases in GSH. These changes coincided with increased DNA fragmentation (Comet assay) in brain tissue, with associated pathologies, including edema, hyalinized axon, and an increased number of inflammatory cells between neurons. The tested dosage is therapeutically relevant. A 400mg/Kg rat dosage would be equivalent to a dose of ~3.9g in a 60Kg human adult, which is below the maximum recommended dosage of 4 g per 24 hours. The temporality of these events needs additional study, such as when the pathology begins to develop, when biomarkers first begin to deviate from controls, and how long recovery takes after exposure to APAP as a function of acute versus chronic durations of exposure. Nevertheless, the results from this study indicate that given the described timing, dose, and duration of exposure, APAP causes oxidative stress, DNA damage, and brain pathologies.

Koehn 2020

This research examined whether *in utero* APAP exposure caused changes in gene expression and whether it had an effect on permeability of the blood-brain barrier.[363] The investigators administered APAP (IP) twice daily to Sprague-Dawley rats from embryonic day E15 to E19 (chronic) or as a single dose at E19 (acute) at 3.75mg/Kg or 15mg/Kg. The authors comment that "[t]he doses used were within the clinically

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[361] Blecharz-Klin et al. Hypothalamus - Response to early paracetamol exposure in male rats offspring. Int J Dev Neurosci. 2019 Aug;76:1-5. doi: 10.1016/j.ijdevneu.2019.05.004. Epub 2019 May 21. PMID: 31125683.

[362] Motawi et al. Protective effects of betanin against paracetamol and diclofenac induced neurotoxicity and endocrine disruption in rats. Biomarkers. 2019 Nov;24(7):645-651. doi: 10.1080/1354750X.2019.1642958. Epub 2019 Aug 5. PMID: 31305161.

[363] Koehn et al. Effects of paracetamol (acetaminophen) on gene expression and permeability properties of the rat placenta and fetal brain. F1000Res. 2020 Jun 8;9:573. doi: 10.12688/f1000research.24119.2. PMID: 32934805; PMCID: PMC7477648.

recommended range (0.5g to 4g in 24 hours in adults)," but the investigators omitted rat-human dose conversion. The doses of 3.75mg/Kg and 15mg/Kg correspond to a HED of about 37.5mg and 150mg in a 60Kg adult, which is well below the therapeutic dose.

On GD19, dams were treated, followed by surgical collection of fetal brains. Fetal and maternal plasma and cerebrospinal fluid were collected and tested for a-fetoprotein and interleukin 1β (IL1β). Labeled 14C-sucrose was used to examine placental permeability. The results showed down-regulation of three acute phase plasma proteins (α-fetoprotein, transferrin, transthyretin) with acute and chronic treatments and up-regulation of immune-response related genes, particularly cytokines, with the chronic treatment. The amount of IL1β in the blood of treated fetuses increased, but no IL1β was found in the controls or in the maternal blood of APAP treated animals. The authors comment that their data "confirms that paracetamol was indeed eliciting an inflammatory response but only on the fetal side of the placental circulation." [364] There was no evidence of increased blood-brain barrier permeability in the fetal brain. The authors suggest that APAP can induce an immune-inflammatory-like response in the placenta if used during pregnancy.

3. Studies Examining Acetaminophen, Glutathione Depletion, and Oxidative Stress

Rat studies were conducted to examine the impact of APAP on glutathione levels and toxicity from oxidative stress.

Stark (1989)

This study showed that the depletion of GSH in cultured rat embryos potentiates the developmental toxicity of APAP.[365] Metabolites of APAP, including NAPQI were also shown to cause disruption of neural tube closure and to decrease viability of embryos.

Stark (1990)

This study characterized APAP in relation to NAPQI and various APAP-analogs using rat embryo culture.[366] Direct additions of APAP to the cultured embryos produced an increased incidence of neural tube defects (NTDs), open anterior neuropores. This contrasted with intra-amniotic microinjections of APAP, which failed to produce NTDs. The APAP metabolite NAPQI produced NTDs and reduced viability when added to the culture directly or via intra-amniotic microinjections. The authors conclude that the metabolic conversion of APAP by the yolk sac (the tissue surrounding the embryo) was associated with NTDs.

Weeks (1990)

[364] Koehn et al. Effects of paracetamol (acetaminophen) on gene expression and permeability properties of the rat placenta and fetal brain. F1000Res. 2020 Jun 8;9:573, p.24. doi: 10.12688/f1000research.24119.2. PMID: 32934805; PMCID: PMC7477648.

[365] Stark et al. Influence of electrophilic character and glutathione depletion on chemical dysmorphogenesis in cultured rat embryos. Biochem Pharmacol. 1989 Aug 15;38(16):2685-92. doi: 10.1016/0006-2952(89)90555-8. PMID: 2764988.

[366] Stark et al. Dysmorphogenesis elicited by microinjected acetaminophen analogs and metabolites in rat embryos cultured in vitro. J Pharmacol Exp Ther. 1990 Oct;255(1):74-82. PMID: 2213573.